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Does inflammation induced by UVB and heat rekindling alter pain related behaviour in rats?

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Running title: UVB/HR model and the burrowing test

Abstract

Objective To investigate whether induction of the UVB and heat rekindling model (UVB/HR) alters burrowing behaviour in rats.

Study design Randomised, blinded, prospective experimental study.

Animals 16 adult male Wistar rats weighing 250-300g.

Methods In the UVB/HR group (n=8), UV irradiation was delivered to the heel area of the right plantar hind paw at a dose of 1000 mJ/cm^2, using a narrowband UVB light source. Twenty four hours later heat rekindling was performed by placement of a feedback controlled thermode set at a constant temperature of 45°C over the area of UVB irradiation for five minutes. Both interventions were carried out under pentobarbital anaesthesia. The SHAM group (n=8) was anaesthetised only. In the burrowing test, rats were singly housed for two hours in cages furnished with a burrow filled with sand. The amount of sand remaining in the burrow after 2 hours was weighed and the amount of sand displaced from the burrow calculated. The burrowing test was carried out for two consecutive days prior to UVB irradiation (day 0) and on day 1 prior to HR and on days 2 and 3 after UVB exposure and at equivalent time points in the SHAM group.

Results Rats in the SHAM group burrowed a mean (SD) of 2429 (73) g and 2358 (124) g of sand on day -2 and day 3 respectively, while in the UVB/HR group the amount of sand burrowed was 2460 (26) and 2419 (58) g on day -2 and day 3 respectively. There was no significant effect of treatment on the amount of sand burrowed at any time point.

Conclusions and clinical relevance Pain associated with UVB/HR model induction is below the threshold required to affect rat burrowing behaviour and therefore questions the face validity of UVB/HR as a translational model of inflammatory pain.

Keywords burrowing, heat rekindling, pain, rat, UVB,

Introduction

The slow progress in analgesic drug development has been attributed to the failure of current animal pain models to replicate the underlying aetiopathogenesis of clinical chronic pain conditions (Yassen et al. 2013). A second factor is reliance on non-innate evoked responses as readouts of analgesic efficacy in animal models, rather than using endpoints that are more relevant to man (Rice et al. 2008; Mogil 2009).

One strategy to address these deficiencies has been the development of translational pain models that can be applied to both animals and humans in analgesia trials. One such model is the Ultraviolet B (UVB) inflammatory pain model, which has been translated from humans to rats and is well characterised in both species (e.g. Gustorff et al. 2004; Bishop et al. 2010; Davies et al. 2011; Gustorff et al. 2013; Mørch et al. 2013; Rukweid et al. 2013; Weerasinghe et al. 2014). The UVB model has recently been extended to include heat rekindling (HR) of the site of UVB exposure (UVB/HR model) and shown to cause a more robust secondary mechanical hyperalgesia in both the human (O'Neill et al. 2013) and rat (O'Neill et al. 2013; Weerasinghe et al. 2014).

The reliance on using evoked responses as readouts of analgesic efficacy has been addressed by the development of behavioural pain biomarkers that utilize innate and ethologically relevant behaviours that are presumed to be more sensitive to changes in pain perception and analgesia. One such biomarker is the burrowing test (Deacon 2006; Deacon 2009), in which the willingness of rodents to burrow in a substrate is measured and taken as a proxy indicator of global wellbeing. Studies have investigated burrowing in rodents

following the induction of inflammatory (Andrews et al. 2011; Andrews et al. 2012; Rutten et al. 2014a; Rutten et al. 2014b), neuropathic (Andrews et al. 2011; Andrews et al. 2012) and surgical (Jirkof et al. 2014) pain and reported reduced burrowing following pain induction that was restored by analgesic administration (Andrews et al. 2011; Andrews et al. 2012; Rutten et al. 2014a; Rutten et al. 2014b). The burrowing test is postulated to be a sensitive method of detecting unprovoked pain and discomfort in rodents.

Unprovoked pain is a significant consequence of chronic pain conditions in man that is associated with a reduced quality of life. Therefore, although undesirable from an animal welfare perspective, unprovoked pain in a translational pain model is a prerequisite for good face validity. Unprovoked pain has not been previously reported in the UVB or UVB/HR model in the rat or human (Bishop et al. 2010 (rat); Gustorff et al. 2013 (human)), although behavioural experiments that might allow the detection of subtle signs of unprovoked pain have not been carried out in animals.

The study aim was to measure the performance of rats in the burrowing test before and after induction of the UVB/HR model, with the hypothesis that rats would be less willing to burrow in the substrate following model induction if inflammation induced by the model was associated with unprovoked pain.

Methods

Animals

Experiments adhered to the IASP Committee for Research and Ethical Issues guidelines as set out by Zimmermann (1983) and were performed in accordance with the UK Animals (Scientific Procedures) Act. A power calculation was not carried out before the start of the study because preliminary data were not available. However previous studies investigating the effect of pain induction on performance in the burrowing assay have utilized seven to ten animals per group and shown a statistically significant decrement in burrowing (Jirkof et al. 2014; Rutten et al. 2014a; Rutten et al. 2014b).

Adult male Wistar rats (250-300g Harlan, Shardlow, UK) were used for all procedures and were kept in pairs within enriched laboratory cages containing transparent plastic hides and cotton rope. Food and water were available *ad libitum* and lights were on from 02:00-14:00. One animal from each cage was identified with non-toxic tattoo paint and permanent marker pen prior to being randomly assigned to either the UVB/HR treatment (n=8) or Sham (anaesthesia only, n=8) group. Randomisation was achieved by pulling one of two different coloured needles out of a bag, with each rat allocated to a needle colour and the selected animal allocated to the UVB/HR treatment group. Animals were anaesthetised using intraperitoneal pentobarbital (45 mg kg^{-1}, pentobarbital sodium salt C-II, Sigma, MO, USA) prior to UVB and heat rekindling procedures.

UVB Irradiation & Heat Rekindling

UV irradiation in the UVB range (290-320 nm) was delivered to the heel area of the right plantar hind paw at a dose of 1000 mJ/cm^2, using a narrowband UVB light source (TLO1 tubes; Phillips, Guildford, UK, λ_{max} = 314 nm) as described in detail by Weerasinghe et al.

(2014). Twenty four hours later heat rekindling was performed by placement of a feedback controlled thermode (built in house) set at a constant temperature of 45°C over the area of UVB irradiation for a duration of five minutes. This UVB and heat rekindling paradigm has been previously shown to produce robust ipsilateral secondary mechanical hyperalgesia without concurrent skin damage (Davies et al. 2011; Weerasinghe et al. 2014).

Behavioural testing

Mechanical withdrawal thresholds

A single experimenter, not blinded to treatment group, measured mechanical withdrawal thresholds on the plantar surface of the right hind paw in the secondary area, on the day of UVB irradiation (day 0, before induction of anaesthesia) and 4 days later. This was to confirm the presence of secondary hyperalgesia in the UVB/HR group. Rats in the Sham group did not undergo measurement of withdrawal threshold because similar Sham treatment did not cause hyperalgesia in previous studies (Davies et al. 2011; Weerasinghe et al. 2014). Rats were placed in individual transparent boxes so that they were in auditory and visual contact with each other, on a raised wire mesh and were allowed to habituate to the environment for 10 minutes before tests. Punctate mechanical stimuli were delivered using calibrated von Frey filaments (North Coast Medical, California (CA), USA) applied to the midplantar region of the hindpaw in the secondary area of uninjured tissue surrounding the primary lesion site on the heel. Different forces of mechanical stimulation were delivered in an order that was devised to minimise induction of mechanical hypersensitivity so that the von Frey filaments that applied more force (26g and 60g) were applied last. Each filament was applied five times for approximately 5 seconds and the

presence or absence of a withdrawal response was recorded as a binary end point. The maximum force delivered to each animal was the minimal weight that yielded a 100% withdrawal response or the cut-off weight of 60g.

Burrowing assay

The burrowing assay was carried out by a single investigator who was blinded to treatment allocation. Burrows were built from sloping, blind-ended plastic tubes 300mm long and 100mm in diameter, elevated at the open end by 60mm as described by Deacon (2006). The weight of each burrow was recorded using a digital scale and then the burrow was filled with 2500g of horticultural grit sand (Cadbury Garden and Leisure, Congresbury, UK). This substrate was used because initial experiments indicated that the Wistar rats burrowed gravel much less vigorously than previously described in Lister rats (Deacon 2006), and studies suggested rats burrow sand more readily than gravel (Deacon 2009). Starting eight days prior to UVB irradiation, rats were habituated to the test apparatus by placement of a filled burrow into their home cage for four consecutive days (days -7 to day -4), though the majority of rats burrowed readily even on initial exposure. Rats were not exposed to the burrows in their home cage on day -3 prior to UVB irradiation.

To carry out the burrowing assay, one filled burrow was placed in a test cage, identical in dimensions to the home cage (480 × 375 × 210 mm), containing a thin layer of sawdust bedding, food pellets and water. One rat was placed in each test cage for two hours at 16.00 hours (two hours into the dark cycle); at the end of the two hour period the rat was returned to its home cage, the burrow immediately weighed and the mass of sand that was displaced from the burrow was calculated. Four rats were tested at any one time in four individual test

cages, so that during the two hour period of the burrowing assay rats were in auditory and visual contact with each other.

The burrowing assay was carried out on day -2 and day -1 prior to UVB exposure, on day 1 post UVB irradiation prior to the heat rekindling procedure, and on days 2 and 3 after UVB exposure. On day 4 rats entered a separate acute experiment carried out under terminal anaesthesia.

Statistical Analyses

Statistical analyses were carried out using Prism 6 for Mac OSX (GraphPad Software, CA, USA). The force at which the animal withdrew 50% of the time (EC_{50}) for mechanical withdrawal threshold was calculated by plotting individual stimulus response curves, where the x-axis displayed log of von Frey filament weight (g) and the y-axis displayed the % withdrawal response as the number of times out of the five applications of the von Frey filament that a withdrawal response occurred (bottom of curve: 0% and top of curve: 100%). A non-linear regression sigmoidal curve was fitted to the plotted data and EC_{50} was measured. EC_{50} data were assessed for normality using a D'Agostino & Pearson omnibus normality test and found to be normally distributed. EC_{50} data were compared between day 0 and day 4 using a paired t-test.

Burrowing data (amount of sand removed from the burrow during each test) were assessed for normality using a Kolmogorov-Smirnov test and found to be normally distributed. Data were analysed using 2-way ANOVA followed by a Tukey post hoc test.

All statistical tests were performed on raw data and are presented as mean ± SD. Statistical significance was defined as $p \leq 0.05$.

Results

UVB irradiation and heat rekindling induced an ipsilateral secondary hyperalgesia to punctate mechanical stimuli (Figure 1)

A significant decrease in EC_{50} mechanical withdrawal threshold was observed on day 4 after UVB irradiation compared with mechanical withdrawal thresholds measured before UVB irradiation (day 0) when testing in an area of secondary hyperalgesia on the ipsilateral paw (Figure 1, P = 0.0084; paired t test). The mean EC_{50} baseline withdrawal threshold was reduced from 23.2 ± 4.7g to 13.9 ± 10.0 g on day 4, equating to a 59.9% reduction in withdrawal threshold.

Performance in the burrowing assay was not altered by UVB irradiation and heat rekindling (Figure 2)

All rats, irrespective of treatment group, burrowed almost all of the sand from the burrow on each of the test days within the 2 hour test period. A 2-way repeated measures ANOVA demonstrated a significant effect of time (day of testing) (P=0.019) on the amount of sand removed from the burrows but no significant effect of treatment (UVB irradiation and heat rekindling (n=8) or Sham (n=8)) and no significant interaction (P =0.89). However, although overall there was a significant effect of time on the amount of sand burrowed, the post hoc Tukey test failed to show significance at any individual time point (Figure 2, two way repeated measures ANOVA).

Discussion

Unprovoked pain is a major contributor to pain unpleasantness in human chronic pain sufferers (Baron et al. 2010; Bennett 2012) and is often poorly amenable to analgesic treatment (Gilron et al. 2013), therefore animal pain models that cause unprovoked pain are desirable for analgesic drug development (Mogil 2009). Unprovoked pain is not easy to measure in animals. Methods rely on the assumption that unprovoked pain will alter the normal behavioural repertoire of an animal and include measurement of altered home cage behaviour, weight bearing during spontaneous movement and altered motivated movement such as wheel running (for a review see Cobos and Portillo-Salido 2013).

Burrowing is a spontaneous innate behaviour performed by laboratory rats that is easy to measure objectively by weighing the amount of substrate that remains in a burrow at the end of the test period. Decreased burrowing is exhibited by rats following induction of inflammatory (Andrews et al. 2011; Andrews et al. 2012; Rutten et al. 2014a; Rutten et al. 2014b) and neuropathic (Andrews et al. 2011; Andrews et al. 2012) pain and restored by analgesic administration (Andrews et al. 2011; Andrews et al. 2012; Rutten et al. 2014a; Rutten et al. 2014b). It is postulated that willingness to burrow is a biomarker of the global wellbeing of a rat and that reduced burrowing behaviour related to pain induction is indicative of reduced wellbeing that accompanies a persistent pain state (Deacon 2006). Reduced burrowing behaviour was also associated with behavioural dysfunction in mice induced by hippocampal lesions (Deacon 2005), supporting the contention that burrowing is indicative of the global wellbeing of a rodent. A previous study in rats showed the burrowing test to be as sensitive as the weight bearing assay and more sensitive than the

open field test at detecting pain caused by sub-chronic inflammation induced by intra-articular injection of Complete Freud's Adjuvant into the knee joint (Rutten et al. 2014a). Administration of analgesic drugs with concurrent sedative properties will tend to reduce burrowing behaviour, a directional change that will not falsely elevate the analgesic effect of drugs with concurrent sedative properties. Although decreased burrowing does not confirm the presence of ongoing, unprovoked pain; it is highly suggestive of a decreased motivation to perform the behaviour that is likely caused by ongoing pain.

Rats in both the UVB/HR and Sham group burrowed almost all of the sand from the burrows during the two hour test on all of the assessment days, with no decrement in burrowing found after exposure to UVB or HR in the treatment group. This finding suggests that the UVB/HR model does not induce ongoing, unprovoked pain in rats, consistent with the lack of spontaneous pain reported in people (Gustorff et al. 2013). Bishop et al. (2010) using an *in vitro* preparation also failed to detect increased spontaneous activity in A delta or C fiber nociceptors following induction of the UVB model compared with activity in naïve skin, although the effects of HR subsequent to UVB exposure were not investigated. Basal spinal c*fos* concentrations were also not increased 48 hours after UVB exposure in rats (Bishop et al. 2007), in support of a lack of ongoing pain. Similarly, the UVB/HR treated rats in the present study did not show obvious behavioural differences in their home cage to Sham rats, supporting the contention that any ongoing pain, if present, was insufficient to cause gross changes in behaviour such as foot licking or paw lifting.

Despite the absence of a change in behaviour in the burrowing test, secondary mechanical hyperalgesia was detected in the UVB/HR group at day 4 after UVB exposure. Behavioural testing with von Frey filaments to detect mechanical hyperalgesia was not carried out prior to day 4 in order to limit exogenous disturbances to the rats during the days when the burrowing test was carried out and it is possible, although unlikely, that the lack of change in the burrowing assay was because the UVB/HR exposure did not cause sensory changes in rats on test days in this experiment. However, to counter this argument, there is very good evidence in rats to indicate that although peak secondary hyperalgesia occurs 72-96 hours after UVB exposure in the UVB/HR model (Davies et al. 2011; Weerasinghe et al. 2014), changes in sensory processing can be detected behaviourally both the day after UV exposure before HR and 48 hours after UVB exposure, 24 hours after HR (Davies et al. 2011). The fact that behavioural changes indicative of altered sensory processing were detected using von Frey filaments, while there was no concurrent change in performance in the burrowing assay in the UVB/HR group indicates that the two behavioural assays are measuring different components of the pain experience. Behavioural testing using von Frey filaments only detects hyperalgesia to a mechanical stimulus and is a measure of the sensory component of evoked pain. In contrast, motivation to burrow is a measure of the impact of pain on the global wellbeing of the animal (Deacon 2006). Thus it is possible to induce pain states where sensory changes are present in the absence of accompanying changes in well being, as documented in the present study.

It is perhaps not surprising that the UVB/HR model in rats did not induce a change in burrowing behaviour that would have been indicative of altered rat wellbeing and ongoing, unprovoked pain given that spontaneous pain is not reported in the model in people. In this

respect the model in rats maintains face validity with its human counterpart. However, given the importance of non-evoked pain in human pain conditions (Baron et al. 2010) this finding does have implications for the usefulness of the model to test the efficacy of new analgesic molecules in both human and animal. It suggests that the model is better suited for the study the underlying aetiopathogenesis of inflammatory pain rather than as a model to test the efficacy of new analgesic drugs that might be effective in the management of ongoing, unprovoked pain.

As stated previously, a sample size of seven to ten animals was chosen based on previously published literature (Jirkof et al. 2014; Rutten et al. 2014a; Rutten et al. 2014b). The fact that almost all sand was burrowed from the burrows in the assay at every test point also suggests that increasing animal numbers would not have altered the findings of the study. Previous studies utilizing the burrowing assay in rats have used gravel as the burrowing substrate, therefore in pilot studies gravel was also used. However, it was quickly apparent that Wistar rats were very reluctant to burrow in gravel during the training phase; when the substrate was changed to sand the rats burrowed very quickly and readily. No distinct learning phase where burrowing increased over the initial exposure days was detected. The time of day that the burrowing test was carried out in the present experiment was also different to previous studies in rats. Andrews et al. (2012) did not report the time that the test was carried out relative to the light:dark cycle to which the rats were habituated, but others have tested rats during the light phase of the day-night cycle (Rutten et al. 2014a; Rutten et al. 2014b) when rats would be expected to be less active than during the dark phase (Tallett et al. 2009). We decided to allow rats access to the burrows during the dark

phase of their day-night cycle in order to optimise activity and therefore burrowing behaviour, particularly because Wistar rats have a reputation for being behaviourly quiescent compared to some other rat species (Clemens et al. 2014). There was an overall effect of time on the amount of substrate burrowed by both groups of rats. The burrowing assay was always carried out at the same time each day therefore this is most likely attributable to the effect of anaesthesia rather than an innate diurnal variation in activity. Shortening the length of time that the rats had access to the burrows may also have facilitated discrimination between treatment groups; 2 hours was allowed because this time period has been used in previous studies.

In accordance with the principle of refinement Von Frey filament thresholds were not measured in the SHAM treatment group because previous studies have shown the SHAM treatment used in this study not to be associated with hypersensitivity to mechanical stimuli (Davies et al. 2011; Weerasinghe et al. 2014). However von Frey data can be very variable between animals and between tests within each animal, therefore the lack of an internal control group that also underwent von Frey testing could also be considered a weakness of the study.

In conclusion, the UVB/HR model did not alter the performance of rats in the burrowing assay indicating that inflammatory pain associated with model induction is below the threshold required to significantly effect the global wellbeing of rats. This has implications for the use of the model to test the efficacy of new analgesic molecules for the treatment of unprovoked, ongoing pain.

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Authors' contributions

All authors contributed to the design of the study and discussed the most appropriate methods for data analysis. MS carried out the experimental work in conjunction with JM, CT, NW and SK. MS analysed the data and JM took a lead role in writing the manuscript in conjunction with BL.

Figure 1 UV-B irradiation induced secondary mechanical hyperalgesia to punctate stimuli. All mechanical stimuli were applied to the midplantar area (area of secondary hyperalgesia) on the ipsilateral paw exposed to UVB irradiation and heat rekindling (n=8). The force at which the animal withdrew from the von Frey filament 50% of the time (EC_{50}) was significantly reduced on day 4 after UVB and heat rekindling compared to day 0 (before UVB irradiation) (P = 0.0084, paired t test). Data are presented as mean ± SD.

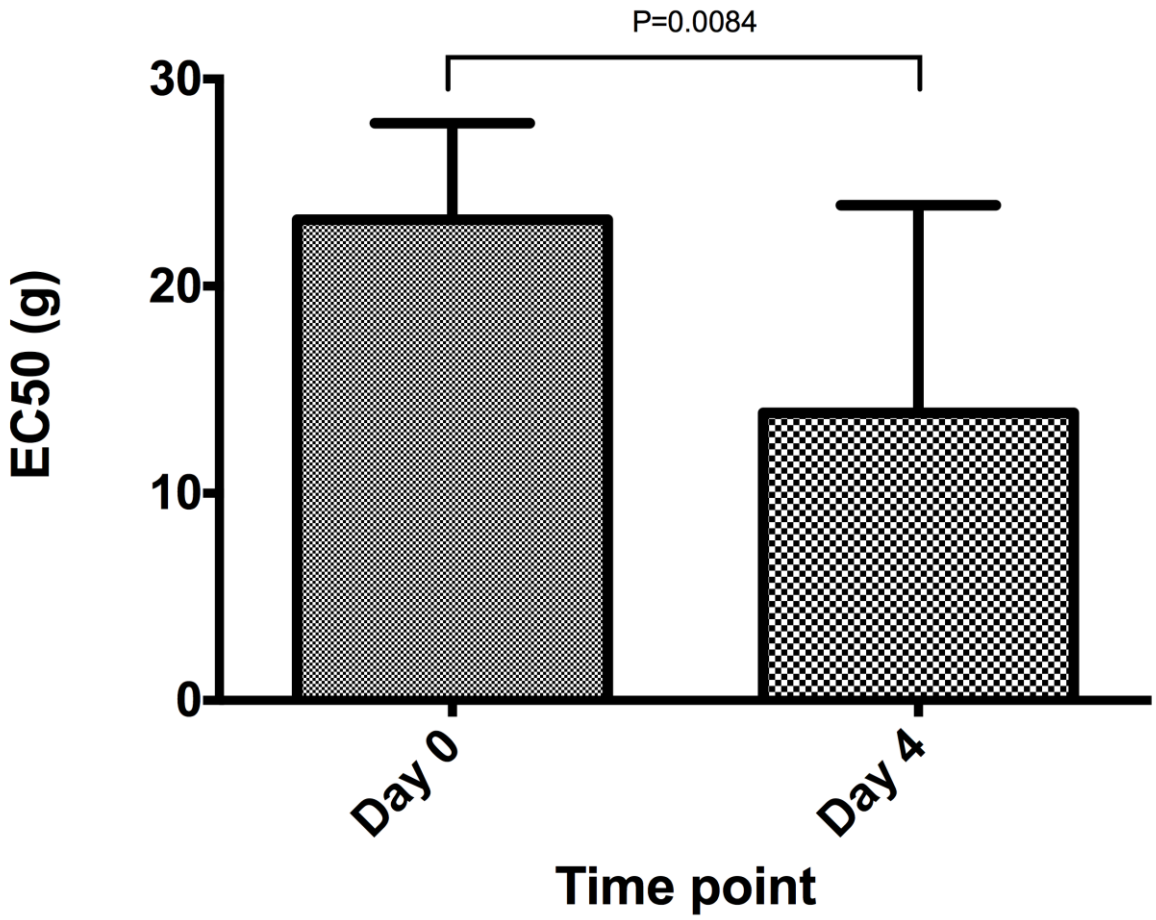
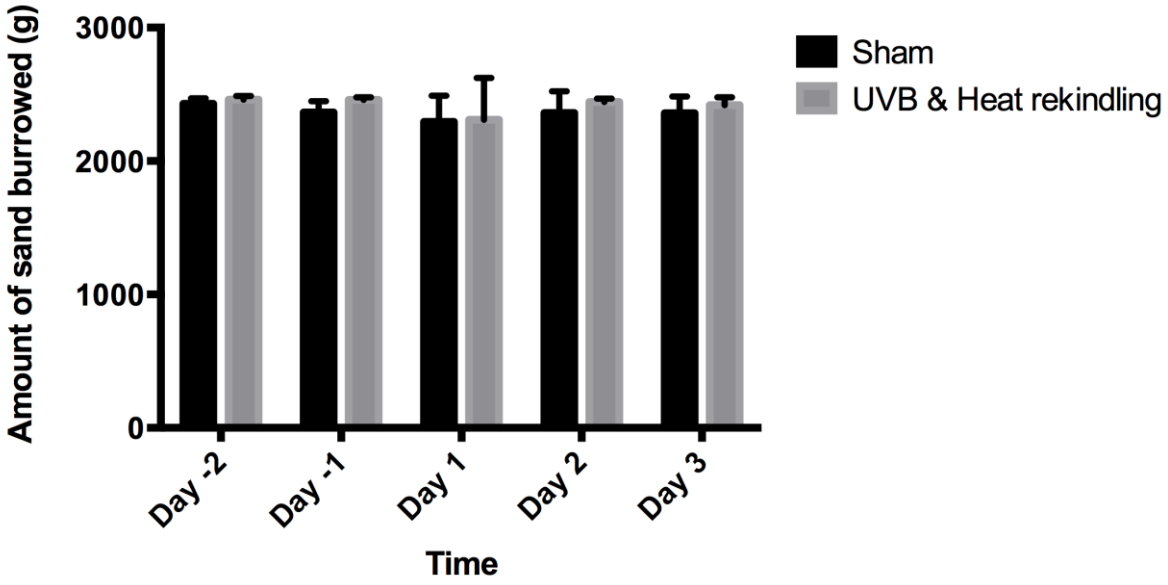


Figure 2: Rats in the UV and heat rekindling (n=8) and Sham (n=8) group burrowed almost all of the sand from the burrow during the 2 hour test period on each day. There was a significant effect of time (P =0.019, two way repeated measures ANOVA), but no

450 significant effect of treatment and no significant interaction. Data are presented as mean ±

451 SD.



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